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(21) International Application Number: PCT/US98/18577 (22) International Filing Date: 4 September 1998 (04.09.98) (30) Priority Data: 60/058,271 8 September 1997 (08.09.97) US 60/060,520 30 September 1997 (30.09.97) US 9722884.5 30 October 1997 (30.10.97) GB 9806032.0 20 March 1998 (20.03.98) GB (71) Applicant (for all designated States except US): MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). (72) Inventor; and (75) Inventor/Applicant (for US only): WILKINSON, Hilary [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). (74) Common Representative: MERCK & CO., INC.; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).			(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, HR, HU, ID, IL, IS, JP, KG, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TJ, TM, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: ESTROGEN RECEPTOR (57) Abstract This invention relates to a novel estrogen receptor and to the polynucleotide sequences encoding this receptor. This invention also relates to methods for identifying ligands which bind to this receptor, to the ligands so identified, and to pharmaceutical compositions comprising such ligands. This invention also relates to pharmaceutical compositions useful for treating or preventing estrogen receptor mediated diseases or conditions, such as abnormal bone resorption, cardiovascular diseases, cancer, or central nervous system disorders.			

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TITLE OF THE INVENTION
ESTROGEN RECEPTOR

CROSS-REFERENCE TO RELATED APPLICATIONS

- 5 This application claims priority to U.S. Provisional Patent Applications Serial No. 60/060,520, filed September 30, 1997, and Serial No. 60/058,271, filed September 8, 1997, both now abandoned.

FIELD OF THE INVENTION

- 10 This invention relates to a novel estrogen receptor and to the polynucleotide sequences encoding this receptor. This invention also relates to methods for identifying ligands which bind to this receptor, to the ligands so identified, and to pharmaceutical compositions comprising such ligands. This invention also relates to pharmaceutical
15 compositions useful for treating or preventing estrogen receptor mediated diseases or conditions.

BACKGROUND OF THE INVENTION

- 20 Nuclear receptors are a large class of proteins that are responsible for the regulation of complex cellular events including cell differentiation, homeostasis, the growth and functioning of various organs and tissues, and transcription. It is believed that nuclear receptors function by transducing extracellular chemical signals from hormones into a transcriptional response.

- 25 Estrogen receptors are a subclass of the larger nuclear receptor class. The estrogen receptors are proteins that are responsive to estrogen and estrogen-like molecules. Estrogen receptors are believed to play an important role in the mammalian endocrine system, the reproductive organs, breast tissue, bone tissue, and the vascular system,
30 and are believed to be involved in the development and progression of various disease states such as abnormal bone resorption, cardiovascular disease, cancer, and central nervous system disorders. It is believed that various disease states and conditions can be treated or prevented by the development of appropriate ligands, i.e. drugs, for modifying the
35 activity of estrogen receptors. Consequently there is a need to identify

estrogen receptors and their mode of action and to also identify ligands for modifying the action of these receptors.

At least two distinct types of estrogen receptors have been reported. An estrogen receptor having 595 amino acids is disclosed in
5 Green, S. et al., *Nature*, 320, pp. 134-139 (1986) and Greene, G.L. et al., *Science*, 231, pp. 1150-1154 (1986), both of which are incorporated by reference herein in their entirety. These references also disclose the corresponding DNA sequences for the receptor.

The other reported type of estrogen receptor has been disclosed by
10 two research groups and has been designated " β " (beta). One research group discloses a 485 amino acid β receptor that is obtained from rat, human, and mouse sources, as well as the corresponding DNA sequences. See PCT application No. WO 97/09348, to Kuiper, G.G. J. M. et al., published March 13, 1997, which is incorporated by reference
15 herein in its entirety. The second research group discloses a similar estrogen receptor containing 483 amino acids. The corresponding DNA sequence is also disclosed. See Mosselman, S. et al., *ER β : identification and characterization of a novel human estrogen receptor*, *FEBS Letters*, 392, pp. 49-53 (1996), which is incorporated by reference herein in its
20 entirety.

In the present invention, a novel estrogen receptor having 548 amino acid units, and that is distinct from the disclosed 595 amino acid, 485 amino acid, and 483 amino acid estrogen receptors, has been
25 identified and isolated from human tissue. It is believed that this novel estrogen receptor plays a key role in mammalian physiology. This novel estrogen receptor is an important research tool for identifying and designing ligands for use in pharmaceutical compositions for treating and/or preventing a wide range of estrogen receptor mediated diseases or conditions.

30 It is therefore an object of the present invention to provide a novel isolated estrogen receptor.

It is another object of the present invention to provide the amino acid sequence of a novel estrogen receptor.

35 It is another object of the present invention to provide the polynucleotide sequence encoding a novel estrogen receptor.

It is another object of the present invention to provide methods for isolating a novel estrogen receptor.

It is another object of the present invention to provide ligands capable of binding to a novel estrogen receptor.

5 It is another object of the present invention to provide pharmaceutical compositions comprising ligands capable of binding to a novel estrogen receptor.

It is another object of the present invention to provide methods for treating and/or preventing estrogen receptor mediated diseases or
10 conditions.

These and other objects will become readily apparent from the detailed description which follows.

SUMMARY OF THE INVENTION

15 The present invention relates to an isolated estrogen receptor comprising the amino acid sequence of Figure 1 (which also corresponds to SEQ ID NO: 1).

In further embodiments, the present invention relates to an isolated estrogen receptor having an amino acid sequence that is
20 substantially similar to the amino acid sequence of Figure 1, wherein the estrogen receptor comprises at least 531 amino acids.

In further embodiments, the present invention relates to an isolated estrogen receptor comprising at least 531 amino acids and
25 having substantially the same ligand binding properties or substantially the same DNA binding properties as the estrogen receptor of Figure 1.

In further embodiments, the present invention relates to an isolated estrogen receptor that is derived from mammalian cells, preferably human cells.

In further embodiments, the present invention relates to an
30 isolated polynucleotide encoding the estrogen receptor having the amino acid sequence of Figure 1.

In further embodiments, the present invention relates to an isolated polynucleotide which is a DNA, a cDNA, or an RNA.

In further embodiments, the present invention relates to an
35 isolated polynucleotide which hybridizes to and is complementary to the

polynucleotide encoding the estrogen receptor having the amino acid sequence of Figure 1.

5 In further embodiments, the present invention relates to an isolated polynucleotide comprising a polynucleotide encoding a mature polypeptide encoded by the estrogen receptor polynucleotide contained in an ATCC Deposit selected from the group consisting of ATCC Deposit No. 209238, ATCC Deposit No. 209239, and ATCC Deposit No. 209240.

10 In further embodiments, the present invention relates to an isolated polynucleotide comprising the nucleotide sequence of Figure 2 (which also corresponds to SEQ ID NO: 2).

In further embodiments, the present invention relates to an isolated polynucleotide which hybridizes to and is complementary to the polynucleotide of Figure 2, wherein said polynucleotide comprises at least 1593 nucleotides.

15 In further embodiments, the present invention relates to a vector containing the DNA.

In further embodiments, the present invention relates to a host cell transformed or transfected with the vector of the present invention.

20 In further embodiments, the present invention relates to a method for producing an estrogen receptor of the present invention.

In further embodiments, the present invention relates to a method for determining whether a ligand can bind to the estrogen receptor of the present invention.

25 In further embodiments, the present invention relates to a ligand detected by the methods of the present invention.

In further embodiments, the present invention relates to a pharmaceutical composition comprising a ligand of the present invention.

30 In further embodiments, the present invention relates to a method for treating or preventing an estrogen receptor mediated disease or condition by administering an effective amount of a pharmaceutical composition of the present invention.

35 The deposits referred to herein will be maintained under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purposes of Patent Procedure. These deposits

are provided merely as a convenience and are not an admission that a deposit is required under 35 USC §112. The sequence of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are
5 incorporated herein by reference in their entirety and are controlling in the event of any conflict with the description of the sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

10 All percentages and ratios used herein, unless otherwise indicated, are by weight. The invention hereof can comprise, consist of, or consist essentially of the essential as well as optional ingredients, components, and methods described herein.

BRIEF DESCRIPTION OF THE FIGURES

15 FIG. 1 shows the amino acid sequence of the estrogen receptor, i.e. the polypeptide, of the present invention.

FIG. 2 shows the nucleotide sequence, i.e. the cDNA polynucleotide, encoding the estrogen receptor of the present invention. This sequence includes the translation termination codon "TGA".

20

DESCRIPTION OF THE INVENTION

In accordance with one aspect of the present invention, there is provided a polypeptide, namely an estrogen receptor, which has the deduced amino acid sequence of FIG. 1 or which has the amino acid
25 sequence encoded by the cDNA of the clone deposited as ATCC Deposit No. 209238 on September 8, 1997, by the genomic DNA of the clone deposited as ATCC Deposit No. 209239 on September 8, 1997, or by the genomic DNA of the clone deposited as ATCC Deposit No. 209240 on September 8, 1997. The present invention also relates to fragments, analogs and derivatives of such an estrogen receptor.

30

The terms "fragments", "derivatives", and "analogs" when referring to the estrogen receptor of FIG. 1 or that encoded by the deposited DNA, means a polypeptide which retains essentially the same biological function or activity as such estrogen receptor. Thus, an

analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature estrogen receptor.

The estrogen receptor of the present invention can be a recombinant polypeptide, a natural polypeptide, or a synthetic polypeptide of the sequence of FIG. 1, or of that encoded by the deposited DNA. Also contemplated within the scope of the present invention are splice variants of the receptor of FIG. 1, or that encoded by the deposited DNA.

The fragments, derivatives, or analogs of the estrogen receptor of FIG. 1 or that encoded by the deposited DNA can be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue can be one that is or is not encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature estrogen receptor is fused with another compound, such as a compound to increase the half-life of the estrogen receptor (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature estrogen receptor, such as a leader or secretory sequence or a sequence which is employed for purification of the mature estrogen receptor or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The present invention also encompasses estrogen receptors which have substantially the same amino acid sequence as the estrogen receptor of Figure 1. In further embodiments of the present invention, the isolated estrogen receptor comprises at least 531 amino acid units and is at least about 75% identical with the sequence shown in Figure 1. In even further embodiments of the present invention, the isolated estrogen receptor comprises at least 531 amino acid units and is at least about 90% identical with the sequence shown in Figure 1. In even further embodiments of the present invention, the isolated estrogen receptor comprises at least 531 amino acid units and is at least about 95% identical with the sequence shown in Figure 1. In even further embodiments of the present invention, the isolated estrogen receptor

comprises at least 531 amino acid units and is at least about 99% identical with the sequence shown in Figure 1.

5 The present invention also encompasses estrogen receptors comprising at least 531 amino acids and having substantially the same ligand binding properties or substantially the same DNA binding properties as that of the estrogen receptor of Figure 1. In other words, the respective ligand binding or DNA binding domains of the receptors have at least about 75% homology, preferably about 90% homology, more preferably about 95% homology, and most preferably about 99% homology to each of the respective ligand binding and DNA binding domains in the receptor of Figure 1.

10 In accordance with another aspect of the present invention, there is provided an isolated nucleic acid, i.e. the polynucleotide, which encodes for the mature estrogen receptor having the deduced amino acid sequence of FIG. 1, or for the mature estrogen receptor encoded by the DNA of the deposited clones.

15 A polynucleotide encoding an estrogen receptor of the present invention can be obtained by performing polymerase chain reactions (PCR) on human testis cDNA and subcloning into a vector in JM109 *E. coli*. Alternatively, the polynucleotide can be obtained by screening a human genomic DNA library derived from human testis.

20 The polynucleotide of the present invention can be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA can be double-stranded or single-stranded, and if single stranded can be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature estrogen receptor can be identical to the coding sequence shown in FIG. 2 or that of the deposited clones or can be a different coding sequence, which coding sequence, as a result of redundancy or degeneracy of the genetic code, encodes the same, mature estrogen receptors as the DNA of FIG. 2 or the deposited DNA.

25 The polynucleotide which encodes for the mature estrogen receptor of FIG. 1 or for the mature polypeptide encoded by the deposited DNA can include: only the coding sequence for the mature polypeptide; 30 the coding sequence for the mature polypeptide and additional coding

sequence such as a leader or secretory sequence or a proprotein sequence; or the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of FIG. 1 or the polypeptide encoded by the DNA of the deposited clones. The variant of the polynucleotide can be a naturally occurring allelic variant of the polynucleotide. The present invention also relates to polynucleotide probes constructed from the polynucleotide sequence of FIG. 2 or a segment of the sequence of FIG. 2 amplified by the PCR method, which can be utilized to screen a cDNA library to deduce the estrogen receptor of the present invention.

Thus, the present invention includes polynucleotides encoding the same mature estrogen receptor as shown in FIG. 1 or the same mature polypeptide encoded by the DNA of the deposited clones, as well as variants of such polynucleotides which variants encode for fragments, derivatives or analogs of the polypeptide of FIG. 2 or the polypeptide encoded by the DNA of the deposited clones. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide can have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in FIG. 2 or of the coding sequence of the deposited clones. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which can have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The present invention further relates to polynucleotides which hybridize to the polynucleotides encoding the estrogen receptor having the amino acid sequence of FIG. 1. The present invention relates to an isolated polynucleotide which hybridizes to and is at least about 75% complementary to the polynucleotide encoding the estrogen receptor having the amino acid sequence of FIG. 1. The present invention relates to an isolated polynucleotide which hybridizes to and is at least about 90% complementary to the polynucleotide encoding the estrogen receptor having the amino acid sequence of FIG. 1. The present invention relates to an isolated polynucleotide which hybridizes to and is at least about 95% complementary to the polynucleotide encoding the estrogen receptor having the amino acid sequence of FIG. 1. The present invention relates to an isolated polynucleotide which hybridizes to and is at least about 99% complementary to the polynucleotide encoding the estrogen receptor having the amino acid sequence of FIG. 1.

The present invention relates to an isolated polynucleotide comprising at least 1593 nucleotides. The present invention relates to an isolated polynucleotide comprising at least 1593 nucleotides which hybridizes to and is at least about 75% complementary to the polynucleotide of FIG. 2. The present invention relates to an isolated polynucleotide comprising at least 1593 nucleotides which hybridizes to and is at least about 90% complementary to the polynucleotide of FIG. 2. The present invention relates to an isolated polynucleotide comprising at least 1593 nucleotides which hybridizes to and is at least about 95% complementary to the polynucleotide of FIG. 2. The present invention relates to an isolated polynucleotide which hybridizes to and is at least about 99% complementary to the polynucleotide of FIG. 2.

The polynucleotides which hybridize to the hereinabove described polynucleotides encode estrogen receptors which retain substantially the same biological function or activity as the mature estrogen receptors encoded by the cDNA of FIG 2 or the deposited DNA. Hybridization is described in U.S. Patent No. 5,501,969, to Hastings et al., issued March 26, 1996, which is incorporated by reference herein in its entirety.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

5 The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally-occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or DNA or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such
10 polynucleotide could be part of a vector and/or such polynucleotide or polypeptide could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically
15 engineered with vectors of the invention and the production of estrogen receptors of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which can be, for example, a cloning vector or an expression vector. The vector can be, for
20 example in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified for activating promoters, selecting transformants or amplifying the estrogen receptor genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host
25 cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotide of the present invention can be employed for producing a polypeptide by recombinant techniques. Thus, for example, the polynucleotide sequence can be included in any one of a variety of
30 expression vehicles, in particular vectors or plasmids for expressing an estrogen receptor. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40: bacterial plasmids; phage DNA; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as
35 vaccinia, adenovirus, fowl, pox virus, and pseudorabies. However, any

other plasmid or vector can be used as long as it is replicable and viable in the host.

As hereinabove indicated the appropriate DNA sequence can be inserted into the vector by a variety of procedures. In general, the DNA
5 sequence is inserted into appropriate restriction endonuclease sites by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The present invention also includes recombinant constructs comprising one or more of the sequences as broadly defined herein. The
10 constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available.

In a further embodiment, the present invention relates to host
15 cells containing the above-described construct. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated
20 transfection, or electroporation (Davis, L., Dibner, M., Battey, I., *Basic Methods in Molecular Biology*, 1986, which is incorporated by reference herein in its entirety).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence.
25 Alternatively, the estrogen receptors of the present invention can be synthetically produced by conventional peptide synthesizers.

Mature estrogen receptors can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such
30 estrogen receptors using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition (Cold Spring Harbor, NY, 1989), which is incorporated by reference herein in its
35 entirety.

The estrogen receptors of the present invention can be naturally purified products expressed from a high expressing cell line, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by
5 bacterial, yeast, higher plant, insect and mammalian cells in culture). Alternatively, a baculovirus/insect cell expression system can also be employed.

The estrogen receptors, their fragments or other derivatives or analogs thereof, or cells expressing them can be used as an immunogen
10 to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain and humanized antibodies, as well as Fab fragments, or the product of a Fab expression library. Various procedures known in the art can be used for the production of such
15 antibodies and fragments.

The present invention is also directed to ligands, i.e. drugs, of the estrogen receptors herein. The term "ligand" as used herein means any molecule which binds to the estrogen receptor of the present invention. These ligands can have either agonist, partial agonist, antagonist,
20 partial antagonist, inverse agonist, or mixtures of these properties. Thus, for example, a ligand that binds to an estrogen receptor of the present invention might modify, inhibit, or eliminate its function. In this way, the ligand can be used to treat or prevent a disease in which the estrogen receptor is involved. The ligands contemplated herein are
25 those that have selectivity to specifically activate or inhibit genes that are normally regulated by the estrogen receptors of the present invention.

The present invention also relates to methods for determining whether a ligand not known to be capable of binding to a human estrogen receptor can bind to a human estrogen receptor. These
30 methods comprise contacting a mammalian cell comprising an isolated DNA molecule encoding a human estrogen receptor with the ligand under conditions permitting binding of ligands known to bind to an estrogen receptor, detecting the presence of any of the ligand bound to a human estrogen receptor, and thereby determining whether the ligand
35 binds to a human estrogen receptor. In these methods, the mammalian

cell is actually expressing the isolated DNA molecules. The general methodology for conducting such a method is well known to those of ordinary skill in the art. See EP 787,797, to Weinshank et al., published July 6, 1997, which is incorporated by reference herein in its entirety.

- 5 Alternatively, RNA that ultimately encodes for the estrogen receptor could be injected into, for example *Xenopus* oocytes, and expressed, and used in analogous assay experiments.

The present invention also relates to pharmaceutical compositions comprising the ligands of the present invention. Such compositions
10 comprise a pharmaceutically effective amount of the ligand. The term "pharmaceutically effective amount", as used herein, means that amount of the ligand that will elicit the desired therapeutic effect or response when administered in accordance with the desired treatment regimen. The ligand is typically administered in admixture with
15 suitable pharmaceutical diluents, excipients, or carriers, collectively referred to herein as "carrier materials", suitably selected with respect to the mode of administration, i.e. oral, I.V., nasal, parenteral, ocular, etc. A wide variety of product and dosage forms well known to one of ordinary skill in the art can be used to administer these ligands.

20 The present invention also relates to methods for treating and/or preventing estrogen receptor mediated diseases or conditions. By "estrogen receptor mediated diseases or conditions" is meant a physiological or pathological state in which an estrogen receptor is involved. Nonlimiting examples of estrogen receptor mediated diseases
25 or conditions include those of the endocrine system, the reproductive organs, breast tissue, bone tissue, and the vascular system, especially those diseases that become more prevalent in aging males and females. More specifically, such diseases and conditions include those selected from the group consisting of abnormal bone resorption, cardiovascular
30 disease, cancer, metabolic disorders, and central nervous system disorders. Even more specifically, such diseases and conditions include those selected from the group consisting of osteoporosis, breast cancer, uterine cancer, ovarian cancer, prostate cancer, diabetes, and Alzheimer's disease.

35

EXAMPLES

The following examples further describe and demonstrate embodiments within the scope of the present invention. The examples are given solely for the purpose of illustration and are not to be construed as limitations of the present invention as many variations thereof are possible without departing from the spirit and scope of the invention.

EXAMPLE 1

Cloning and Sequencing of cDNA Clones of a Human Estrogen Receptor Gene

The 5' rapid amplification of cDNA ends (RACE) product was identified by performing two rounds of polymerase chain reactions (PCR) on human testis Marathon-Ready cDNA (Clontech product #7414-1) using Vent Polymerase (New England Biolabs product #254S). The first round of PCR was performed using the oligonucleotide, GGAGAAAGGTGCCCAGGTGTTGGCC (SEQ ID NO: 3), in the 5' coding region of human estrogen receptor beta (GenBank sequence number X99101) and the Clontech AP1 primer, according to the manufacturer's instructions. The second round of PCR was performed using either of two different nested primers having the sequences GTGGTCTGCCGACCAGGCCACC (SEQ ID NO: 4) or GGTGTTGGCCACAACACATTTGG (SEQ ID NO: 5), corresponding to the 5' end of a human estrogen receptor beta clone (GenBank sequence number X99101), and the Clontech AP2 primer, according to the manufacturer's instructions. The PCR product was subcloned into the PCRampScript vector (Stratagene product # 211188) in JM109 E. coli. This clone was sequenced on both strands by cycle sequencing (Pharmacia product #27-1694-01), according to the manufacturer's instructions using primers corresponding to the vector sequence having the following sequence GTAATACGACTCACTATAGGGC (SEQ ID NO: 6) as well as a primer in the 5' end of the human estrogen receptor beta receptor gene having the following sequence GTTAGTGACATTGCTGGGAATGC (SEQ ID NO: 7). Further sequencing was performed with four additional primers having the

following sequences: GATCAGAGGCTTCAGCGAAACAG (SEQ ID NO: 8), GAACGCGTGGATTAGTGACTAGCC (SEQ ID NO: 9), GGAGGAAGGAGAATTAAGGCTAG (SEQ ID NO: 10), and GAGATAACAGCTGAGAAAACACC (SEQ ID NO: 11). These four primers were derived from the initial sequence analysis. Sequence alignments and analysis of the nucleotide and protein sequences were carried out using MacVector and AssemblyLign programs (Oxford Molecular Group) as well as the GCG Sequence Analysis Software Package (Madison, WI: pileup).

10

EXAMPLE 2

Cloning and Sequencing of Genomic DNA Clones of a Human Estrogen Receptor Gene

15 To obtain a probe for use in the screening of a human genomic DNA library, cDNA was first generated from human testis mRNA (Clontech product #6535-1) using an oligo-dT primer and MMLV Reverse Transcriptase (Stratagene product #200420) according to the manufacturer's instructions. The cDNA was amplified by PCR using
20 Boehringer Mannheim's Expand High Fidelity PCR System (product #1 732 641) and two primers having the following sequences: GTGATGAATTACAGCATTCCCAGCAATGTCACTAACTTGGGAAGG (SEQ ID NO: 12) and
25 ATGGCCCAAGCTTGGGTTCAGTTCACCTCAGGGCCAGGCG (SEQ ID NO: 13). The PCR product was cloned into the TGEM vector (Promega product #A3600) in JM109 *E. coli*. The product was sequenced on one strand with a Pharmacia cycle sequencing kit (product #27-1694-01) according to the manufacturer's instructions using nine primers having the following sequences: CTTGGAAGGTGGGCCTGGTCGGC
30 (SEQ ID NO: 14), GGAGAAAGGTGCCAGGTGTTGGCC (SEQ ID NO: 15, which is identical to SEQ ID NO: 3), CCGTTGCGCCAGCCCTGTTACTGG (SEQ ID NO: 16), CGCAAGAGCTGCCAGGCCTGCCG (SEQ ID NO: 17), CCCCAGCAGCTAGTGCTCACCC (SEQ ID NO: 18),
35 CTTGGAGAGCTGTTGGATGGAGG (SEQ ID NO: 19),

CTCTGTGTCAAGGCCATGATCC (SEQ ID NO: 20),
CGTCAGGCATGCGAGTAACAAGGG (SEQ ID NO: 21), and
GCAAGTCCTCCATCACGGGGTCCG (SEQ ID NO: 22), corresponding
to the published DNA sequence (Mosselman, S. et al., *ERβ: identification
and characterization of a novel human estrogen receptor, FEBS Letters*,
392, pp. 49-53 [1996]). Sequence alignments and analysis of the
nucleotide and protein sequences were carried out using MacVector and
AssemblyLign programs (Oxford Molecular Group) as well as the GCG
Sequence Analysis Software Package (Madison, WI: pileup).

The cDNA clone obtained was digested with the restriction
enzymes NcoI and KpnI to obtain an approximately 500 base pair
fragment corresponding to the 5' end of the human estrogen receptor
beta cDNA (GenBank sequence number X99101). This fragment was
labeled with P-32 and used to screen a human genomic DNA library
(Stratagene product #946206) as per the manufacturer's instructions.
One million bacteriophage plaques were screened and seventeen
potential hybridizing phages were chosen. These phages were
reamplified and screened using a slightly smaller probe (i.e an
approximately 300 base pair fragment generated by digesting the human
ERbeta clone with NcoI and PstI). Two positive phages were plaque
purified and used for the production of DNA. The phages were digested
with NotI and BamHI to generate smaller fragments encoding most of
the phage DNA and these were subcloned into pBluescript (Stratagene;
GenBank #52324). There were two fragments from one phage of
approximately 8.5 and 6kb and two fragments from the other phage of
approximately 7.7 and 6.3 kb. The genomic subclones of 8.5 and 7.7 kb
were sequenced on both strands with a Pharmacia cycle sequencing kit
(product #27-1694-01) according to the manufacturer's instructions
using primers derived from the 5'RACE product sequencing (EXAMPLE
1). Sequence alignments and analysis of the nucleotide and protein
sequences were carried out using MacVector and AssemblyLign
programs (Oxford Molecular Group) as well as the GCG Sequence
Analysis Software Package (Madison, WI: pileup).

WHAT IS CLAIMED IS:

1. An isolated estrogen receptor comprising the amino acid sequence of Figure 1.
5
2. An isolated estrogen receptor according to Claim 1 having an amino acid sequence that is at least about 99% identical with the sequence shown in Figure 1, wherein said estrogen receptor comprises at least 531 amino acids.
10
3. An isolated estrogen receptor according to Claim 1 having an amino acid sequence that is at least about 95% identical with the sequence shown in Figure 1, wherein said estrogen receptor comprises at least 531 amino acids.
15
4. An isolated estrogen receptor according to Claim 1 having an amino acid sequence that is at least about 90% identical with the sequence shown in Figure 1, wherein said estrogen receptor comprises at least 531 amino acids.
20
5. An isolated estrogen receptor according to Claim 1 having an amino acid sequence that is at least about 75% identical with the sequence shown in Figure 1, wherein said estrogen receptor comprises at least 531 amino acids.
25
6. An isolated estrogen receptor comprising at least 531 amino acids having substantially the same ligand binding properties as that of the estrogen receptor of Figure 1.
- 30 7. An isolated estrogen receptor comprising at least 531 amino acids having substantially the same DNA binding properties at that of the estrogen receptor of Figure 1.
- 35 8. An isolated estrogen receptor according to Claim 1 that is derived from mammalian cells.

9. An isolated estrogen receptor according to Claim 1 that is derived from human cells.

5 10. An isolated polynucleotide encoding the estrogen receptor having the amino acid sequence of Figure 1.

11. The polynucleotide of Claim 10 wherein the polynucleotide is DNA.
10

12. The DNA of Claim 11 wherein the DNA is a cDNA.

13. An isolated polynucleotide which hybridizes to and is at least about 99% complementary to the polynucleotide of Claim 11.
15

14. An isolated polynucleotide which hybridizes to and is at least about 95% complementary to the polynucleotide of Claim 11.

15. An isolated polynucleotide which hybridizes to and is at least about 90% complementary to the polynucleotide of Claim 11.
20

16. An isolated polynucleotide which hybridizes to and is at least about 75% complementary to the polynucleotide of Claim 11.

25 17. An isolated polynucleotide comprising a polynucleotide encoding a mature polypeptide encoded by the estrogen receptor polynucleotide contained in an ATCC Deposit selected from the group consisting of ATCC Deposit No. 209238, ATCC Deposit No. 209239, and ATCC Deposit No. 209240.

30

18. An isolated polynucleotide comprising the nucleotide sequence of Figure 2.

19. An isolated polynucleotide which hybridizes to and is at least about 99% complementary to the polynucleotide of Claim 18, wherein said polynucleotide comprises at least 1593 nucleotides.
- 5 20. An isolated polynucleotide according to Claim 19 wherein said polynucleotide is a cDNA.
21. An isolated polynucleotide which hybridizes to and is at least about 95% complementary to the polynucleotide of Claim 18, 10 wherein said polynucleotide comprises at least 1593 nucleotides.
22. An isolated polynucleotide according to Claim 21 wherein said polynucleotide is a cDNA.
- 15 23. An isolated polynucleotide which hybridizes to and is at least about 90% complementary to the polynucleotide of Claim 18, wherein said polynucleotide comprises at least 1593 nucleotides.
24. An isolated polynucleotide according to Claim 23 wherein 20 said polynucleotide is a cDNA.
25. An isolated polynucleotide which hybridizes to and is at least about 75% complementary to the polynucleotide of Claim 18, 25 wherein said polynucleotide comprises at least 1593 nucleotides.
26. An isolated polynucleotide according to Claim 25 wherein said polynucleotide is a cDNA.
27. A vector containing the DNA of Claim 11.
- 30 28. A host cell transformed or transfected with the vector of Claim 27.
29. A method for producing an estrogen receptor comprising:

expressing from the host cell of Claim 28 the estrogen receptor encoded by said DNA.

5 30. A method for determining whether a ligand not known to be capable of binding to a human estrogen receptor can bind to a human estrogen receptor, said method comprising:

- 10 (a) contacting a mammalian cell, comprising an isolated DNA molecule encoding a human estrogen receptor having the amino acid sequence of Figure 1, with the ligand under conditions permitting binding of ligands known to bind to an estrogen receptor,
- (b) detecting the presence of any of the ligand bound to a human estrogen receptor, and
- 15 (c) determining whether the ligand binds to a human estrogen receptor.

31. A ligand detected by the method of Claim 30.

- 20 32. A pharmaceutical composition comprising:
- (a) a ligand identified by the method of Claim 30, and
- (b) a pharmaceutically acceptable carrier.

25 33. A method for treating or preventing an estrogen receptor mediated disease or condition, said method comprising administering an effective amount of a pharmaceutical composition according to Claim 32.

30 34. A method according to Claim 33 wherein said estrogen receptor mediated disease or condition is selected from the group consisting of abnormal bone resorption, cardiovascular diseases, cancer, metabolic disorders, or central nervous system disorders.

35 35. A method according to Claim 33 wherein said estrogen receptor mediated disease or condition is osteoporosis.

36. A method according to Claim 33 wherein said estrogen receptor mediated disease or condition is selected from the group consisting of breast cancer, uterine cancer, ovarian cancer, prostate cancer, diabetes, or Alzheimer's disease.

5

1/2

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FIG.1

2/2

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FIG.2

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/18577

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 14/00, 14/705; C12N 5/16, 15/11; G01N 33/53
US CL :435/69.1, 7.1, 325; 530/350; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1; 7.1, 325; 530/350; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, BIOSCIENCE, CAPLUS, MEDLINE, BIOSIS

search terms: estrogen (5a) receptor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KUIPER et al. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. 11 June 1996, Vol. 93, No. 12, pages 5925-5930, see entire document.	1-30
A	MOSSELMAN et al. ERbeta: identification and characterization of a novel human estrogen receptor. FEBS Letter. 19 August 1996, Vol. 392, No. 1, pages 49-53, see entire document.	1-30

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

01 DECEMBER 1998

Date of mailing of the international search report

14 JAN 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

MICHAEL D. PAK

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/18577

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-30, drawn to an isolated estrogen receptor, an isolated polynucleotide, a vector, a host cell, a method for producing an estrogen receptor, and method for determining a ligand binding.

Group II, claim(s) 31-32, drawn to a ligand and a pharmaceutical composition comprising the ligand.

Group III, claim(s) 33-36, drawn to a method for treating disease.

and it considers that the International Application does not comply with the requirements of unity of invention (Rules 13.1, 13.2 and 13.3) for the reasons indicated below:

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons.

Pursuant to 37 CFR 1.475(d), the claims of group I are considered by the ISA/US to constitute the main invention, and none of the related groups II-III correspond to the main invention.

The product of Group III do not share the same or corresponding special technical feature with Group I, because they are drawn to products having materially different structures and functions, and each defines a separate invention over the art.

The methods of Group III, do not share the same or corresponding special technical feature with Group I, because the product is not used or made by the methods, and each defines a separate invention over the art.

Since Groups I-III do not share a special technical feature, unity of invention is lacking.